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An evaluation of the use of ITS sequences in the taxonomy of the *Hypocreales*

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Abstract: The taxonomic utility of rDNA internal transcribed spacer (ITS) sequences in the *Hypocreales* is assessed. ITS-based phylogenies in *Trichoderma* and *Fusarium* are critically evaluated, with brief comments on other genera. ITS sequences have been successfully used to support teleomorph-anamorph connections and articulate phylogenetic relationships in certain groups of *Trichoderma*. However, they lack sufficient variation for species delineation in *Fusarium*, demonstrating that the ITS region is not universally applicable as a species-level marker. Alternative genes used for species level systematics are mentioned. We discuss the consequences of the occurrence of multiple ITS types in *Fusarium*. In conclusion, we emphasize the importance of a multidisciplinary approach to defining species, an approach that reflects the fungal organism as a whole.

Key words: *Trichoderma, Fusarium*, phylogeny, species concepts, ribosomal DNA

Introduction

The Ascomycete order *Hypocreales* includes common soil fungi, economically important plant pathogens, as well as saprobes in many habitats. Morphologically, the order is characterized partly by the production of lightly coloured perithecial ascomata and unitunicate asci, but many species rarely or never undergo sexual reproduction. They are often found as anamorphs, classified in about 35 anamorph genera (Rossman *et al.*, 1999). Two of the most important anamorph genera allied with the *Hypocreales* are *Fusarium* Link and *Trichoderma* Pers. *Trichoderma* species, linked to the teleomorph genus *Hypocrea* Fr., have commercial applications such as biological control, enzyme production and as a model system for genetics. *Fusarium* species, linked to *Gibberella* Sacc., *Haematonectria* Samuels & Nirenberg, *Cosmospora* Rabenh. and *Albonectria* Rossman & Samuels (Rossman *et al.* 1999), produce potent mycotoxins and cause diseases of grains, vegetables and fruits.

With recent advances in automation of DNA sequencing and computerization of analytical methods, sequence data are now routinely used to infer phylogenies in fungi. An often characterized part of the genome is the internal transcribed spacer region (ITS-1 and ITS-2) of the ribosomal RNA gene cluster. This region is generally considered to encompass variation near the species level (Kohn, 1992) and consequently is often studied as a part of taxonomic revisions of fungal genera. As of this writing, there are about 350 ITS sequences (or partial sequences) available in GenBank for *Trichoderma* spp., about 280 for *Fusarium* spp., about 20 for species of *Balansia* Speg., 14 each for *Cordyceps* (Fr.) Link and *Verticillium* Nees (only some of which pertain to the *Hypocreales*, see Cannon & Kirk, this volume), 12 for species of *Stachybotrys* Corda, 10 each for *Epichloë* (Fr.) Tul., *Neotyphodium* Glenn, Bacon, Price & Hanlin and *Sepedonium* Link, and the remainder for species of other genera. The database is constantly growing, and about 200 sequences were added while we worked on this paper.

The general taxonomic utility of ITS sequences in the *Hypocreales* can now be critically assessed. We will focus on data for *Fusarium* and *Trichoderma* as models because of the preponderance of data on these two genera, commenting on other genera as appropriate.

Trichoderma and *Fusarium* – an overview

The taxonomic problems in *Trichoderma* and *Fusarium* are not unique, but are more obvious because the impact of these fungi on human affairs has stimulated taxonomic research. The main problem is to adequately define generic and species concepts.

Rifai (1969) initiated modern *Trichoderma* taxonomy, replacing the prevailing monotypic taxonomy with one recognizing nine species aggregates based on morphological and physiological characters. This work was refined by Bissett (1984, 1991a, b). The connection of *Trichoderma* to *Hypocrea* dates from Tulasne & Tulasne (1865) and today the phylogenetic equivalence of *Trichoderma* and *Hypocrea* is well-established, although some *de facto* species are apparently strictly asexual and only have species epithets in *Trichoderma*. This equivalence, long suspected based on the preponderance of anamorph–teleomorph connection data, was supported by phylogenetic analysis of molecular data, mainly ITS sequences, and confirmed by RFLP and RAPD analyses (Kuhls *et al.*, 1996; Lieckfeldt *et al.*, 1998). Gams & Bissett (1998) compiled recent results in a revised key recognizing four sections in *Trichoderma*. Contemporary taxonomists working with *Trichoderma* generally cooperate in the sharing of strains and data, resulting in several truly multidisciplinary studies to resolve species-level questions (e.g. Samuels *et al.*, 1998).

Modern *Fusarium* taxonomy dates from Wollenweber & Reinking (1935), the spirit of which is followed in the most recent complete taxonomy by Gerlach & Nirenberg (1982). *Fusarium* taxonomy is complicated by the widespread use of identification guides promoting broad species concepts (at its most extreme Toussoun & Nelson, 1976; to a lesser extent Nelson *et al.*, 1983 and Burgess *et al.*, 1994). Teleomorphs of *Fusarium* are found in four genera of the *Nectriaceae*, an arrangement generally supported by molecular data (cf. O'Donnell, 1993). The taxonomic intricacies of this situation are discussed by Seifert (2000) and Seifert & Samuels (this volume). The practical importance of mycotoxin production by *Fusarium* species means that chemotaxonomic analysis of secondary metabolites has received more attention than in *Trichoderma*, providing a valuable supplementary data set for taxonomic evaluation. However, the continued existence of taxonomic schools has interfered with the development of cooperative international taxonomic studies, with some exceptions (e.g. Nirenberg, 1995).

The structure of the ITS and the occurrence of multiple types in the *Hypocreales*

The ITS consists of two regions of approximately equal length (in the *Hypocreales*), the ITS-1 and ITS-2, separated by the 5.8S rDNA (White *et al.*, 1990.). Waalwijk *et al.* (1996) first recognized the occurrence of two different ITS-2 types in a broad range of *Fusarium* species. They noted that although analysis of ITS-1 sequences of thirteen species resulted in a reliable phylogeny, the ITS-2 analysis divided species into two distantly related groups that did not correlate with other phylogenetic hypotheses. O'Donnell & Cigelnik (1997) noticed the same phenomenon, and designed PCR primers allowing retrieval of each ITS-2 type in single strains, which occurred as major or minor ITS constituents in most of the tested species of the *Gibberella fujikuroi* (Sawada) Ito clade (Fig. 2; O'Donnell *et al.*, 1998a). The occurrence of divergent ITS-2 types may be the result of an ancient gene duplication, or interspecific hybridization event, which occurred before the divergence of the *G. fujikuroi* clade. In a practical sense, the occurrence of divergent ITS types in *Fusarium* means that RFLP studies based on this region need to be interpreted with considerable care (cf. Donaldson *et al.*, 1995).

We have surveyed for multiple ITS-2 types in other genera of the *Hypocreales* using the Type I and Type II primers designed by O'Donnell & Cigelnik (1997). We obtained preliminary evidence for the occurrence of two ITS-2 types in *Gliocladium polyporicola* (Henn.) Seifert, Samuels & W. Gams (DAOM 216041), *'Nectria' mariannaeae* Samuels & Seifert [anamorph: *Mariannaea* cf. *elegans* (Corda) Samson, CBS 746.88] and *Cylindrocarpon destructans* (Zinssm.) Scholten (teleomorph: *'Nectria' radicicola* Gerlach & L. Nilsson, DAOM 221059) (Seifert & Désaulniers, unpublished). However, we have not confirmed this by sequencing amplified DNA products. To our knowledge there are no reports of multiple ITS types in *Trichoderma*, but this has not been investigated in detail.

In *Trichoderma*, the ITS-1 is the more variable of the two ITS regions, which is even obvious from ITS-1 length variations among different sections. Members of sections *Longibrachiatum*, *'Pachybasium'* and *Trichoderma* have ITS-1 sequences about

180, 200 and 220 base pairs (bp) long respectively. There are few cases where ITS-1 lengths diverge from these ranges. An example is the 230 bp ITS-1 of the ex-type isolate *T. aureoviride* Rifai, anamorph of *H. aureoviridis* Plowright & Cooke which, according to morphological concepts of Bissett (1991a), should belong to section *Trichoderma*. In *Fusarium*, the total length of the ITS region is relatively constant, varying from 455 to 475 bp, including the 158 bp 5.8S rDNA gene. The length of the ITS-1 varies from 146–149 bp, while the ITS-2 type II is longer (161–167 bp) than ITS-2 type I (151–153 bp) (O'Donnell *et al.*, 1998a). No length variations have been noted within species or different sections of the genus. In species of *Sepedonium* Link, an anamorph genus associated with some species of *Hypomyces* Tul., the ITS-1 is about 189 bp, the 5.8 S about 156 bp, and the ITS-2 about 203 bp (Sahr *et al.*, 1999).

Some studies have limited sequencing of the ITS to either the ITS-1 or ITS-2. In our opinion, a sequence of about 200 bp is too short to recover sufficient informative sites for analysis. Given the ability of modern automated sequencers to produce sequences of 800–1200 bp per primer, the more restricted capacity of manual DNA sequencing (normally 300–400 bp primer) is probably no longer relevant.

Taxonomic studies in the *Hypocreales* using the ITS region

As noted above, the ITS region is generally considered phylogenetically informative near the species level (Kohn, 1992). It is important to remember that cladograms from ITS sequences (or any other single genes) represent gene trees and not species trees (Doyle, 1992). Therefore, the impact of 'pure' ITS data on taxonomy is limited. From a theoretical standpoint, the question of what actually constitutes a species has been associated with the taxonomic resolution of the ITS for about a decade, but more sophisticated phylogenetic species concepts are now changing this view (O'Donnell *et al.*, 1998a; Taylor *et al.*, 1999). From a practical standpoint, the question of whether this region is actually able to reliably provide species signatures is critical for scientists interested in molecular diagnostics. However, outside the *Hypocreales* there are examples of highly divergent ITS sequences within single morphological 'species' [e.g. *Seiridium cardinale* (Wagener) B. Sutton & Gibson with about 15% divergence in the ITS-1 among 12 strains; Viljoen *et al.*, 1993]. Conversely, identical ITS sequences have been determined in complexes of closely related species (e.g. *Sclerotium* Tode; Carbone & Kohn, 1993). Despite these limitations, in ecological niches where a limited number of closely related species occur, ITS data may be useful for developing diagnostic probes or PCR primers. For example, O'Donnell & Gray (1995) designed ITS-based PCR primers for the diagnosis of soybean sudden death syndrome caused by *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli*.

In terms of taxonomic impact, ITS sequences have had more influence on *Trichoderma* (Fig. 1) than on other genera in the *Hypocreales*. They have been used for the delimitation, identification and classification of strains active in biological control (*T. atroviride* P. Karsten, *T. harzianum* Rifai), hyperproducing certain enzymes (*T. reesei* E. G. Simmons) or aggressively competing with other fungi (*T. harzianum sensu lato*). ITS sequences have been included in the description of neotypes of previously known species (*T. harzianum*, *T. atroviride*, *T. koningii* Oud.; Muthumeenakshi *et al.*, 1994, 1998; Gams & Meyer, 1998; Lieckfeldt *et al.* 1998) and in the characterization of new species (*T. asperellum* Samuels, Lieckfeldt & Nirenberg, 1999).

There has been little confusion about the generic concept of *Trichoderma* since its initial description in 1794; there is only one generic synonym, as opposed to *Fusarium* with at least 15 generic synonyms. There has only been one minor extension to the concept of *Trichoderma* to include *T. virens* (J. Miller, Giddens & Foster) von Arx, long considered a species of *Gliocladium*. The original proposal, based on morphological and cultural characters (von Arx, 1987) was later supported by ITS (Muthumeenakshi, 1994) and 28S rDNA sequences (Rehner & Samuels, 1994).

Analysis of relationships among species of *Trichoderma* and separation of species aggregates into constituent species have been difficult. In recent years, molecular methods have clarified sectional and species-level problems and have begun to answer the question of how many species of *Trichoderma* there are. Unlike *Fusarium*, in which hundreds of species have been described at a relatively constant rate for almost 200 years (cf. http://www.cbs.knaw.nl/fusarium), the majority of *Trichoderma* species were described in the last thirty years. Over the past six years, it has been shown that ITS sequences are useful in characterizing strains to the sectional and in some cases species level. Analysis of ITS sequence data demonstrated the monophyly of *Trichoderma* section *Longibrachiatum* (Kuhls *et al.*, 1997). Moreover, single *Trichoderma* species in this section are characterized by unique ITS sequences. Although these differences are limited to a few base pairs, inves-

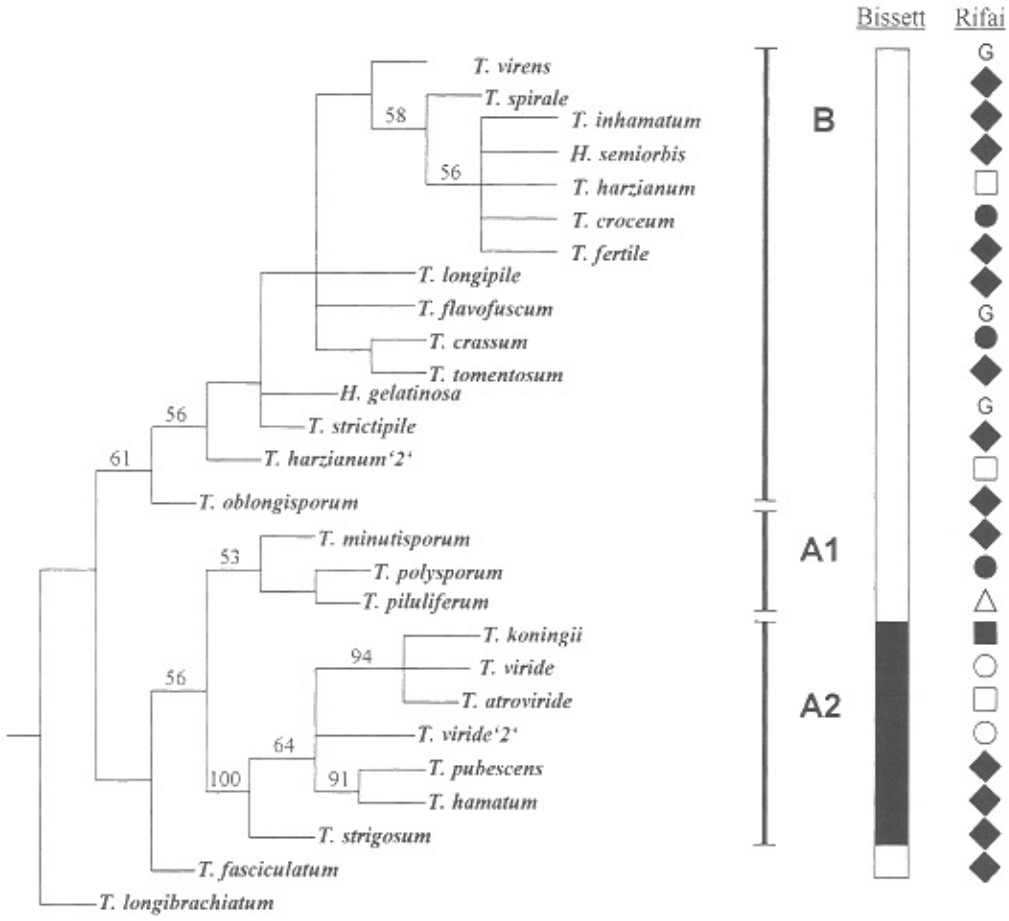

Fig. 1. ITS sequences based phylogeny of ex-type strains in *Trichoderma* sections *Pachybasium* and *Trichoderma*. The vertical bars designated A1, A2 and B refer to clades discussed in Kinderman *et al.* (1998). The bars labeled 'Bissett' denote species attributed to section *Pachybasium sensu* Bissett (white bar) and *Trichoderma* (black bar). The symbols below the label 'Rifai' refer to the species aggregates described by Rifai (1968), namely G = *Gliocladium*, ♦ = *T. hamatum*, □ = *T. harzianum*, ● = *T. polysporum*, ■ = *T. koningii*, ○ = *T. viride*, Δ = *T. piluliferum*. Redrawn from Figure 1 in Kinderman *et al.* (1998). Reproduced with the permission of Academic Press.

investigations of new isolates have confirmed the utility of these species-specific sequences.

However, some delimitations of sections of *Trichoderma* using ITS sequences have been incongruent with previous classifications based on morphology. Phylogenetic analyses suggest that section *Pachybasium sensu* Bissett (1991b) is paraphyletic, with the type of the section, *T. hamatum*, occurring in section *Trichoderma*, away from the monophyletic group representing the remaining species formerly attributed to section *Pachybasium* (referred to elsewhere in this manuscript as section 'Pachybasium' pending the eventual formal renaming of this clade). The species formerly included in section *Pachybasium* comprise two sister clades, one including all *T. polysporum* (Link) Rifai strains and the other including the largest number of all remaining species of the section as defined by morphological and physiological characteristics (Bissett, 1991b). A smaller number of species, namely *T. pubescens* Bissett, *T. strigosum* Bissett, the *T. hamatum* (Bonorden) Bainier neotype, and one of the four *T. harzianum* types described by many authors (Muthumeenakshi *et al.* 1994, 1998; Grondona *et al.* 1997; Ospina-Giraldo *et al.* 1998; Dodd *et al.* 2000), cluster together with section *Trichoderma* (see Fig. 2 in Kindermann *et al.*, 1998). All molecular data (including sequences of ITS, 28S rDNA, endochitinase and RAPDs) supports our conclusion that *Trichoderma* section *Trichoderma* is monophyletic with two well-defined sister clades. In a combined analysis of molecular and morphological data, we demonstrated that section *Trichoderma* is homogeneous (Lieckfeldt *et al.* 1999), but the question arises whether some morphological characters used to delimit species correlate with ITS sequences, for example the mor-

phologically distinct species *T. viride* and *T. koningii*. Studies of more variable parts of the genome may help answer this question (Lieckfeldt *et al.*, 2000). *Hypocrea aureoviridis* (anamorph: *T. aureoviride*) certainly does not belong to this section.

Most taxonomic evaluations of the ITS in *Trichoderma* are based on the whole region, spanning ITS-1–5.8S–ITS-2. In the few studies comparing separate analyses of the two ITS regions (Dodd *et al.*, 2000), there is a tendency for ITS-2 to be more informative for taxa with a somewhat ambiguous position in the gene tree/cladogram, although it is less variable than ITS-1 on a percentage basis. Dodd *et al.* (2000) showed one example where a *T. inhamatum* Veerkamp & W. Gams strain had a different position in trees generated from ITS-1 or ITS-2 data. The authors speculated that this was a result of recombination or hybridization between two of the other taxa under investigation and favoured removing such taxa from the analysis. We have similar cases from our own data, such as *T. ghanense* Doi *et al.* of section *Longibrachiatum*, which is more basal in that section and seems more closely related to sections 'Pachybasium' and *Trichoderma* when considering ITS-2 (Lieckfeldt, unpublished). There is a possibility of different evolutionary rates in the ITS-1 and ITS-2, which implies a need for separate analyses. The partition homogeneity test (Swofford *et al.*, 1996) should be applied to independent ITS-1 and ITS-2 alignments before combined data are analysed.

Early molecular taxonomic studies in *Fusarium* were reviewed by Manicom *et al.* (1990). *Fusarium* was one of the first fungal genera to undergo a comprehensive molecular study, which employed RNA sequencing of the 28S (Guadet *et al.*, 1989). The conclusions were in accordance with observations based on anamorph–teleomorph connections that certain species, in particular the snow mould now called *Microdochium nivale* (Fr.) Samuels & Hallett, were phylogenetically unrelated to most other species of *Fusarium*. By the time DNA sequencing was a routine part of fungal taxonomy in the early 1990's, the generic limits of *Fusarium* were already established in their present state (Gams & Nirenberg 1989). However, the sectional arrangement within the genus, more or less fixed since Wollenweber & Reinking (1935), has been repeatedly challenged by molecular data. For example, sections *Liseola*, *Elegans* and *Dlaminia* are individually paraphyletic, although they collectively form a monophyletic group, which was demonstrated first using sequences of ITS alone (Waalwijk *et al.*, 1996) or in combination with other gene sequences (O'Donnell & Cigelnik, 1997; O'Donnell *et al.*, 1998a).

Early attempts to delineate *Fusarium* species using sequence data, undoubtedly influenced by yeast taxonomy, employed 28S rDNA (Peterson & Logrieco, 1991; Logrieco *et al.*, 1991). The amount of variation was so low between closely related but reproductively isolated species that attention quickly shifted elsewhere. O'Donnell (1992) noted the occurrence of three different ITS types in strains identified as *Gibberella pulicaris* (Fr.) Sacc. (*Fusarium sambucinum* Fuckel). These three types were later shown to correspond to different phylogenetic species in a multidisciplinary taxonomic study known as the European *Fusarium sambucinum* project (Nirenberg, 1995). However, subsequent studies in *Fusarium* have shown that insufficient ITS variation exists to resolve other species complexes. The studies of Waalwijk *et al.* (1996), O'Donnell & Cigelnik (1997) and O'Donnell *et al.* (1998a) have all demonstrated identical ITS sequences for reproductively isolated *Gibberella* species and phylogenetically related *Fusarium* species (species joined on the same vertical lines in Fig. 2).

In other genera of the *Hypocreales*, ITS phylogenies correspond relatively well with morphologically based species concepts. In the *Nectriaceae*, studies of the ITS in *Calonectria* De Not. and *Cylindrocladiella* Boesewinkel result in a gene tree that resolves many species. More variation exists in the ITS-2 between species, and little variation of ITS-1 and ITS-2 sequences exists within species (Schoch *et al.*, this volume). In the *Hypocreaceae*, the ITS gene tree for species of *Sepedonium* correlates well with morphological species. Some variation among strains of some species was noted (Sahr *et al.*, 1999). In the *Clavicipitaceae*, small variations in the ITS of different host-specific populations *Claviceps purpurea* (Fr.) Tul. have been noted, but their taxonomic significance has not yet been assessed (White *et al.*, this volume).

Do the relationships suggested by ITS correlate well with relationships suggested by other rDNA domains or nuclear or mitochondrial genes? For *Trichoderma*, few comparative studies are published. Dodd *et al.* (2000) sequenced the D2 domain of the 28S rDNA along with the ITS-1 and ITS-2 regions of 50 strains representing seven *Trichoderma* species. Eight sequence patterns were resolved, but there was insufficient variability to provide a reliable phylogeny; nevertheless, ITS sequences revealed the same eight groups. Muthumeenakshi *et al.* (1994, 1998) investi-

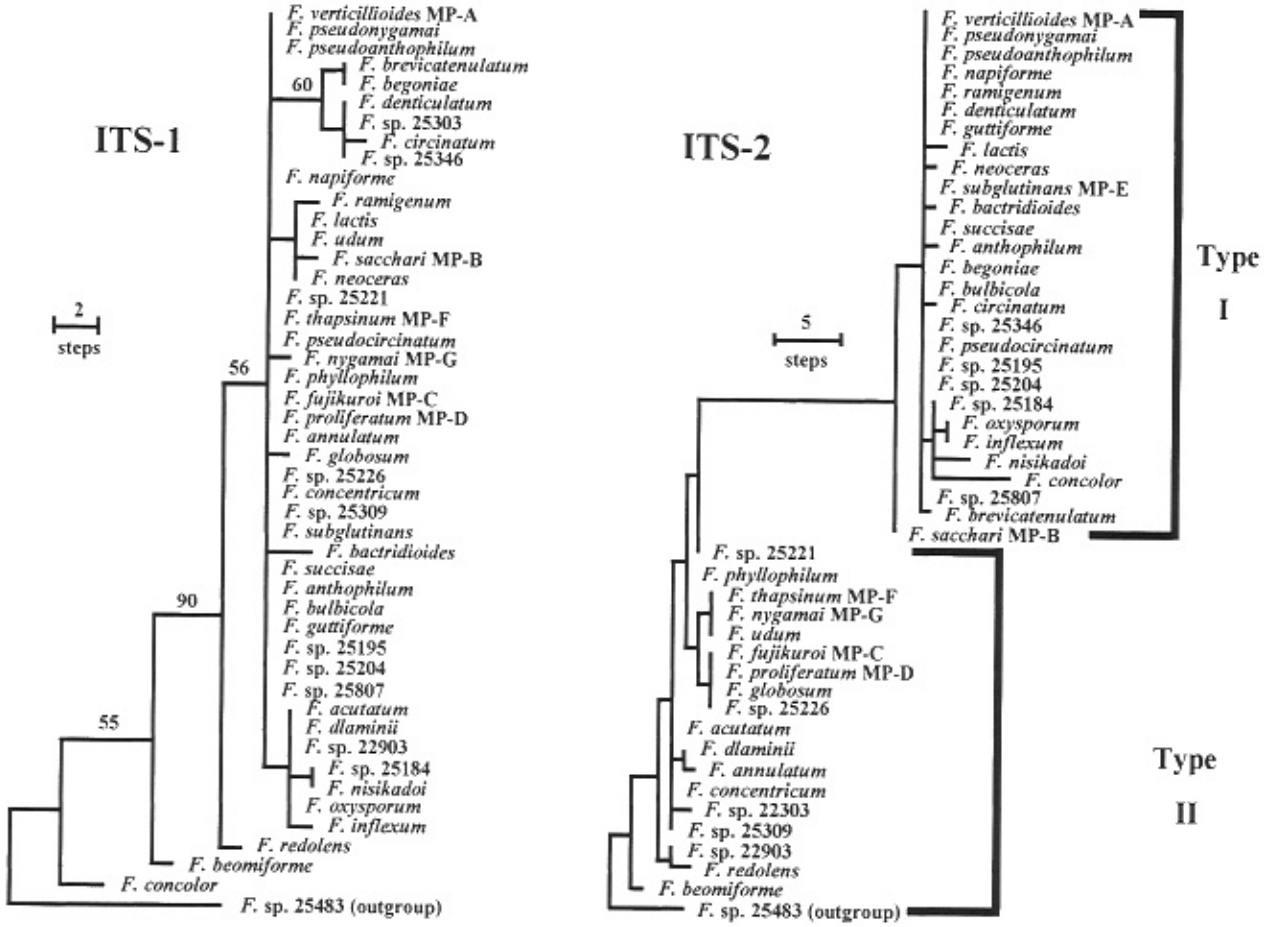

Fig. 2. ITS sequence-based phylogeny of the *Gibberella fujikuroi* complex, showing a relatively poorly resolved species-level phylogeny using the ITS-1 (right) and the even less resolved phylogeny using the type-I and type-II ITS-2 (left). Branches lacking species epithets refer to accession numbers for unidentified cultures in NRRL. From Figure 2 in O'Donnell *et al.* (1998a). Reproduced with the permission of the authors.

gated genetic diversity in the *T. harzianum* complex using RFLPs of mtDNA and ITS-1 sequences and found the same groups with both techniques. For species characterization in section *Trichoderma*, we have compared sequence information from the D1 and D2 domains of the 28S rDNA, ITS-1, ITS-2, and the 42 kDa endochitinase gene. Sequence variability in the D1 and D2 was less than in the ITS, but the two main clades of this section were resolved. RFLPs of the 1450 bp endochitinase gene and sequence comparisons of the first 500 bp clearly resolved the main clades into smaller clades correlating with species defined by morphological characters (Lieckfeldt *et al.*, 2000). In *Fusarium*, the ITS has a similar degree of resolution to the 28S, which is in both cases poor compared to protein-coding genes (O'Donnell *et al.*, 1998). The existence of two ITS-2 types, noted above, further complicates the comparison of ITS and 28S phylogenies.

Until recently, the relationships of anamorphic 'species' with teleomorphs was studied more intently in *Trichoderma* than in *Fusarium*. The first molecular evidence that an asexual *Trichoderma* is a clonal derivative of a species of *Hypocrea* came from the investigation of *T. reesei* and *H. jecorina* by ITS-sequencing and RAPDs (Kuhls *et al.*, 1996). With the characterization of the *Hypocrea schweinitzii* (Fr.) Sacc. complex and *Trichoderma* section *Longibrachiatum* (Kuhls *et al.*, 1997; Samuels *et al.*, 1998), as well as a revision of sections *Trichoderma* including the *Hypocrea rufa* (Pers.) Fr. complex (Lieckfeldt *et al.*, 1998, 1999) and 'Pachybasium' (Kindermann *et al.*, 1998), further connections between *Trichoderma* and *Hypocrea* teleomorphs were established. Six *Trichoderma-Hypocrea* connections are supported by 100% identity in ITS-1 and ITS-2 sequences. In *Fusarium*, apparently anamorphic species are intercalated with sexually competent species (designated as MP-A, MP-B etc. in Fig. 2). More resolved gene trees (e.g. β-tubulin, mitochondrial small rDNA)

suggest sister-group relationships between several anamorph species and teleomorphic species (O'Donnell *et al.*, 1998).

There have been many discussions concerning sequence variability within monophyletic groups, and the question of how many base differences define a species has often been raised. Our present knowledge of the ITS in the *Hypocreales* suggests that tree topology is more critical than the precise number of base pair differences. In *Trichoderma*, there are several species that, according to their current delimitations, have more ITS variation within them than exists between different species in other parts of the genus. It is likely that these species concepts will need to be reconsidered. In *Fusarium*, relatively invariant ITS sequences have led to the sequencing of protein-coding genes, such as β-tubulin, elongation factor α, calmodulin and others, because they reveal the finer taxonomic resolution needed for species-level systematics (O'Donnell *et al.* 1998a, b). In other genera of the *Hypocreales*, the search for phylogenetic markers to complement or to replace ITS sequences is now in progress. In *Calonectria* and *Cylindrocladiella*, Schoch *et al.* (this volume and unpublished) sequenced the β-tubulin (*BenA*) gene and the HMG box of *mat-2* and found tree topologies concordant with but more resolved than ITS data, supporting morphological and biological species concepts in those genera. The newly investigated genes also resolved some geographic populations below the species level. In *Cylindrocarpon destructans*, β-tubulin sequences have revealed considerably more infraspecific variation than published studies of the ITS (Hamelin *et al.*, 1996) suggesting the existence of several phylogenetic species and some host-specific populations (Yee, Seifert & Louis-Seize, unpublished). In the *Clavicipitaceae*, Tsai *et al.* (1994) have also made extensive use of β-tubulin sequences to develop species phylogenies in *Epichloë*.

As species-level systematics progresses, it seems likely that phylogenetic species concepts, based on concordance between five or more gene trees (Taylor *et al.*, 1999), will replace the standard sometimes now accorded to the ITS.

Future prospects using the ITS for taxonomic purposes in the *Hypocreales*

Considering molecular investigations done on *Trichoderma* and *Fusarium*, four general observations can be made: (i) after a short period of testing different molecular methods, sequencing of the ITS regions was chosen as *the* method for delimiting *Trichoderma* species and confirming their infrageneric relationships, whereas (ii) the region has been essentially abandoned in *Fusarium* as other genes with more resolution have been discovered, (iii) more recently, ITS sequence studies have been combined with morphological, biochemical and physiological data, and sequences of other genes and handled in so-called multidisciplinary approaches, and (iv) in both *Trichoderma* and *Fusarium*, sectional classifications are not monophyletic, but these morphologically based concepts retain value to facilitate identification.

The differing taxonomic resolutions of the ITS in *Fusarium* and in parts of *Trichoderma* bring several important problems to the surface. It is clear that the region is not universally applicable as a species-level marker, and may be insufficient to recognize recently evolved or rapidly evolving species. Despite this, the availability of the large ITS database suggests that continued sequencing of this region should serve to anchor data from other more variable, protein-coding genes that may be investigated. ITS sequence variability in *Trichoderma* teaches us that even within small, monophyletic taxonomic groups, species might evolve at different rates. Sequence heterogeneity in section 'Pachybasium' might indicate a higher evolutionary rate in comparison to the overall homogeneity in sections *Longibrachiatum* and *Trichoderma*, which could be related to the action of many strains as pathogens (fungus–host interaction and genetic variation). So, the evolutionary process itself makes it difficult to demarcate species from varieties and to define species limits using any one gene alone. In *Fusarium*, other genes have been used to delve deeper into phylogenetic relationships. It remains to be seen how similar probing will affect the taxonomy of *Trichoderma*.

In 1994, Samuels and Rehner pointed out that, 'We lack an objective measure of the variability of any *Trichoderma* species', a problem that seems universal in developing a reliable taxonomy for most genera of economically important microfungi. Where are we six years later, with all this ITS sequence information? How can the gene trees be most effectively used to develop practical classifications? The problems we face in both *Trichoderma* and *Fusarium* are universal: (i) species definition and (ii) genotype /ecotype/pathotype characterization. Because of the obvious practical needs, there is a tendency to consider these different problems as identical. We conclude by emphasizing the importance of what we have called a multidisciplinary approach to defining species. In our opinion, it is important to use as many characters as possible that reflect the whole organism, including its ecology. Combining different data sets to form cladograms or phenograms presents

as yet unresolved challenges, but there are mathematical models able to perform analyses of complex data sets (e.g. correspondence analysis). With the help of such approaches, we should be able to solve both problems. In the meantime, scientists with different areas of expertise should work with shared groups of rigorously identified strains that can form the basis for a unified species-level taxonomy.

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